PRODUCTION OF GAMMA LINOLENIC ACID BY A Δ6-DESATURASE

This is a continuation-in-part of U.S. Serial No. 08/789,936 filed January 28, 1997, now US Patent 5,789,220 which is a continuation-in-part of U.S. Serial No. 08/307,382, filed September 14, 1994, now US Patent 5,552,306 which is a continuation of U.S. Serial No. 07/959,952 filed October 13, 1992, now abandoned, which is a continuation-in-part of U.S. Serial No. 07/817,919, filed January 8, 1992, now abandoned, which is a continuation-in-part application of U.S. Serial No. 07/774,475 filed October 10, 1991, now abandoned

FIELD OF THE INVENTION

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme Δ6-desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides nucleic acids comprising the Δ6-desaturase gene. More specifically, the nucleic acids comprise the promoters, coding regions and termination regions of the Δ6-desaturase genes. The present invention is further directed to recombinant constructions comprising a Δ6-desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

BACKGROUND OF THE INVENTION

Unsaturated fatty acids such as linoleic ($C_{18}\Delta^{9,12}$) and α-linolenic ($C_{18}\Delta^{9,12,15}$) acids are essential dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between the Δ^9 double bond and the methyl-terminus of the fatty acid chain. Because they are precursors of other products, linoleic and α-linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into γ-linolenic acid (GLA, $C_{18}\Delta^{6,9,12}$) which can in turn be converted to arachidonic acid (20:4), a critically important fatty acid since it is an essential precursor of most prostaglandins.

The dietary provision of linoleic acid, by virtue of its resulting conversion to GLA and arachidonic acid, satisfies the dietary need for GLA and arachidonic acid. However, a relationship has been demonstrated between consumption of saturated fats and health risks such as hypercholesterolemia, atherosclerosis and other clinical disorders which correlate with susceptibility to coronary disease, while the consumption of unsaturated fats has been associated with decreased blood cholesterol concentration and reduced risk of atherosclerosis. The therapeutic benefits of dietary GLA may result from GLA being a precursor to arachidonic acid and thus subsequently contributing to prostaglandin synthesis. Accordingly,

consumption of the more unsaturated GLA, rather than linoleic acid, has potential health benefits. However, GLA is not present in virtually any commercially grown crop plant.

Linoleic acid is converted into GLA by the enzyme Δ6-desaturase. Δ6-desaturase, an enzyme of more than 350 amino acids, has a membrane-bound domain and an active site for desaturation of fatty acids. When this enzyme is transferred into cells which endogenously produce linoleic acid but not GLA, GLA is produced. The present invention, by providing genes encoding Δ6-desaturase, allows the production of transgenic organisms which contain functional Δ6-desaturase and which produce GLA. In addition to allowing production of large amounts of GLA, the present invention provides new dietary sources of GLA.

SUMMARY OF THE INVENTION

The present invention is directed to isolated Δ6-desaturase genes. Specifically, the isolated genes comprise the Δ6-desaturase promoters, coding regions, and termination regions.

The present invention is further directed to expression vectors comprising the Δ6-desaturase promoter, coding region and termination region.

Yet another aspect of this invention is directed to expression vectors comprising a Δ6-desaturase coding region in functional combination with

heterologous regulatory regions, i.e. elements not derived from the Δ6-desaturase gene.

Cells and organisms comprising the vectors of the present invention, and progeny of such organisms, are also provided by the present invention.

A further aspect of the present invention provides isolated bacterial Δ6-desaturase. Isolated plant Δ6-desaturases are also provided.

Yet another aspect of this invention provides a method for producing plants with increased gamma linolenic acid content.

A method for producing chilling tolerant plants is also provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS:

Fig. 1 depicts the hydropathy profiles of the deduced amino acid sequences of Synechocystis Δ6-desaturase (Panel A) and Δ12-desaturase (Panel B). Putative membrane spanning regions are indicated by solid bars. Hydrophobic index was calculated for a window size of 19 amino acid residues [Kyte, et al. (1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75, cSy13 and Csy7 with overlapping regions and subclones. The origins of subclones of Csy75, Csy75-3.5 and Csy7

are indicated by the dashed diagonal lines. Restriction sites that have been inactivated are in parentheses.

Fig. 4 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) tobacco.

Fig. 5A depicts the DNA sequence of a Δ6-desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the open reading frame in the isolated borage Δ6-desaturase cDNA. Three amino acid motifs characteristic of desaturases are indicated and are, in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of the borage Δ6-desaturase to other membrane-bound desaturases. The amino acid sequence of the borage Δ6-desaturase was compared to other known desaturases using Gene Works (IntelliGenetics). Numerical values correlate to relative phylogenetic distances between subgroups compared.

Fig. 7 is a restriction map of 221.Δ6.NOS and 121.Δ6.NOS. In 221.Δ6.NOS, the remaining portion of the plasmid is pBI221 and in 121.Δ6.NOS, the remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography profiles of mock transfected (Panel A) and 221.Δ6.NOS transfected (Panel B) carrot cells. The positions of 18:2, 18:3 α, and 18:3 γ(GLA) are indicated.

Fig. 9 provides gas liquid chromatography profiles of an untransformed tobacco leaf (Panel A) and

a tobacco leaf transformed with 121.Δ6.NOS. The positions of 18:2, 18:3 a, 18:3γ (GLA), and 18:4 are indicated.

Fig. 10 is the complete DNA sequence and deduced amino acid sequence of evening primrose Δ6-desaturase. A heme binding motif of cytochrome *b*5 proteins is indicated by underlined bold text. Underlined plain text indicates three histine rich motifs (HRMs). The motifs in this sequence are identical to those found in borage Δ6-desaturase with the exception of those that are italicized (S 161 and L 374).

Fig. 11 is a formatted alignment of the evening primrose and borage Δ6-desaturase amino acid sequences.

Fig. 12A is a Kyte-Doolittle hydrophobicity plot for borage Δ6-desaturase.

Fig. 12B is a Kyte-Doolittle hydrophobicity plot for evening primrose Δ6-desaturase.

Fig. 13A is a Hopwood hydrophobicity plot for borage Δ6-desaturase. The y axis is a normalized parameter that estimates hydrophobicity; that the x axis represents the linear amino acid sequences.

Fig. 13B is a Hopwood hydrophobicity plot for evening primrose Δ6-desaturase. X and y axes are as in Figure 13A.

Fig. 14A graphically depicts the location of the transmembrane regions for borage Δ6-desaturase. Positive values (y-axis) greater than 500 are considered

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significant predictors of a membrane spanning region. The x-axis represents the linear amino acid sequences.

Fig. 14B graphically depicts the location of the transmembrane regions for evening primrose Δ6-desaturase. X and y axes are as in Figure 14A.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention provides isolated nucleic acids encoding Δ6-desaturase. To identify a nucleic acid encoding Δ6-desaturase, DNA is isolated from an organism which produces GLA. Said organism can be, for example, an animal cell, certain fungi (e.g. Mortierella), certain bacteria (e.g. Synechocystis) or certain plants (borage, Oenothera, currants). The isolation of genomic DNA can be accomplished by a variety of methods well-known to one of ordinary skill in the art, as exemplified by Sambrook et al. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY. The isolated DNA is fragmented by physical methods or enzymatic digestion and cloned into an appropriate vector, e.g. a bacteriophage or cosmid vector, by any of a variety of well-known methods which can be found in references such as Sambrook et al. (1989). Expression vectors containing the DNA of the present invention are specifically contemplated herein. DNA encoding Δ-desaturase can be identified by gain of function analysis. The vector containing fragmented DNA is transferred, for example by infection, transconjugation, transfection, into a host organism

that produces linoleic acid but not GLA. As used herein, "transformation" refers generally to the incorporation of foreign DNA into a host cell. Methods for introducing recombinant DNA into a host organism are known to one of ordinary skill in the art and can be found, for example, in Sambrook et al. (1989). Production of GLA by these organisms (i.e., gain of function) is assayed, for example by gas chromatography or other methods known to the ordinarily skilled artisan. Organisms which are induced to produce GLA, i.e. have gained function by the introduction of the vector, are identified as expressing DNA encoding Δ-desaturase, and said DNA is recovered from the organisms. The recovered DNA can again be fragmented, cloned with expression vectors, and functionally assessed by the above procedures to define with more particularity the DNA encoding Δ6-desaturase.

As an example of the present invention, random DNA is isolated from the cyanobacteria Synechocystis Pasteur Culture Collection (PCC) 6803, American Type Culture Collection (ATCC) 27184, cloned into a cosmid vector, and introduced by transconjugation into the GLA-deficient Cyanobacterium Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, DNA molecules comprising Δ6-desaturase genes have been isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a Δ6-desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding Δ6-desaturase, the 3.588 kb fragment that confers Δ6-desaturase activity is cleaved into two subfragments, each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)] are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are identified as Neo^R green colonies on a brown background of dying non-conjugated cells after two weeks of growth on selective media (standard mineral media BG11N + containing 30μg/ml of neomycin according to Rippka et al., (1979) J. Gen Microbiol. 111, 1). The green colonies are selected and grown in selective

liquid media (BG11N + with 15μg/ml neomycin). Lipids are extracted by standard methods (e.g. Dahmer et al., (1989) Journal of American Oil Chemical Society 66, 543) from the resulting transconjugants containing the forward and reverse oriented ORF1 and ORF2 constructs. For comparison, lipids are also extracted from wild-type cultures of Anabaena and Synechocystis. The fatty acid methyl esters are analyzed by gas liquid chromatography (GLC), for example with a Tracor-560 gas liquid chromatograph equipped with a hydrogen flame ionization detector and a capillary column. The results of GLC analysis are shown in Table 1.

Table 1: Occurrence of C18 fatty acids in wild-type and transgenic cyanobacteria

SOURCE	18:0	18:1	18:2	c-18:3	c18:3	18:4
Anabaena (wild type)	+	+	+	-	+	-
Anabaena + ORF1(F)	+	+	+	-	+	-
Anabaena + ORF1(R)	+	+	+	-	+	-
Anabaena + ORF2(F)	+	+	+	+	+	+
Anabaena + ORF2(R)	+	+	+	-	+	-
Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient Anabaena gain the function of GLA production when the construct containing ORF2 in forward orientation is introduced by transconjugation. Transconjugants

containing constructs with ORF2 in reverse orientation to the carboxylase promoter, or ORF1 in either orientation, show no GLA production. This analysis demonstrates that the single open reading frame (ORF2) within the 1884 bp fragment encodes Δ6-desaturase. The 1884 bp fragment is shown as SEQ ID NO:3. This is substantiated by the overall similarity of the hydropathy profiles between Δ6-desaturase and Δ12-desaturase [Wada et al. (1990) Nature 347] as shown in Fig. 1 as (A) and (B), respectively.

Also in accordance with the present invention, a cDNA comprising a Δ6-desaturase gene from borage (Borago officinalis) has been isolated. The nucleotide sequence of the 1.685 kilobase (kb) cDNA was determined and is shown in Fig. 5A (SEQ ID NO: 4). The ATG start codon and stop codon are underlined. The amino acid sequence corresponding to the open reading frame in the borage delta 6-desaturase is shown in Fig. 5B (SEQ ID NO: 5).

Additionally, the present invention provides a Δ6-desaturase gene from evening primrose (Oenothera biennis). The nucleotide sequence of the 1.687 kb cDNA was determined and is depicted in Figure 10 (SEQ ID NO:26). Also shown in Figure 10 is the deduced amino acid sequence of evening primrose Δ6-desaturase.

Isolated nucleic acids encoding Δ6-desaturase can be identified from other GLA-producing organisms by the gain of function analysis described above, or by nucleic acid hybridization techniques using the isolated

nucleic acid which encodes Synechocystis, borage, or evening primrose Δ6-desaturase as a hybridization probe. Both methods are known to the skilled artisan and are contemplated by the present invention. The hybridization probe can comprise the entire DNA sequence disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or a restriction fragment or other DNA fragment thereof, including an oligonucleotide probe. Methods for cloning homologous genes by cross-hybridization are known to the ordinarily skilled artisan and can be found, for example, in Sambrook (1989) and Beltz et al. (1983) Methods in Enzymology 100, 266.

In another method of identifying a delta 6-desaturase gene from an organism producing GLA, a cDNA library is made from poly-A^+ RNA isolated from polysomal RNA. In order to eliminate hyper-abundant expressed genes from the cDNA population, cDNAs or fragments thereof corresponding to hyper-abundant cDNAs genes are used as hybridization probes to the cDNA library. Non hybridizing plaques are excised and the resulting bacterial colonies are used to inoculate liquid cultures and sequenced. For example, as a means of eliminating other seed storage protein cDNAs from a cDNA library made from borage polysomal RNA, cDNAs corresponding to abundantly expressed seed storage proteins are first hybridized to the cDNA library. The "subtracted" DNA library is then used to generate expressed sequence tags (ETSs) and such tags are used to scan a data base such as GenBank to identify potential desaturates.

Using another method, an evening primrose cDNA may be isolated by first synthesizing sequences from the borage Δ6-desaturase gene and then using these sequences as primers in a PCR reaction with the evening primrose cDNA library serving as template. PCR fragments of expected size may then be used to screen an evening primrose cDNA library. Hybridizing clones may then be sequenced and compared to the borage cDNA sequence to determine if the hybridizing clone represents an evening primrose Δ6-desatuase gene.

Transgenic organisms which gain the function of GLA production by introduction of DNA encoding Δ6-desaturase also gain the function of octadecatetraeonic acid ($18:4^{\Delta 6,9,12,15}$) production. Octadecatetraeonic acid is present normally in fish oils and in some plant species of the Boraginaceae family (Craig et al. [1964] J. Amer. Oil Chem. Soc. 41, 209-211; Gross et al. [1976] Can. J. Plant Sci. 56, 659-664). In the transgenic organisms of the present invention, octadecatetraenoic acid results from further desaturation of α-linolenic acid by Δ6-desaturase or desaturation of GLA by Δ15-desaturase.

The 359 amino acids encoded by ORF2, i.e. the open reading frame encoding Synechocystis Δ6-desaturase, are shown as SEQ. ID NO:2. The open reading frame encoding the borage Δ6-desaturase is shown in SEQ ID NO: 5. The present invention further contemplates other nucleotide sequences which encode the amino acids of SEQ ID NO:2 and SEQ ID NO: 5. It is within the ken of the

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ordinarily skilled artisan to identify such sequences which result, for example, from the degeneracy of the genetic code. Furthermore, one of ordinary skill in the art can determine, by the gain of function analysis described hereinabove, smaller subfragments of the fragments containing the open reading frames which encode Δ6-desaturases.

The present invention contemplates any such polypeptide fragment of Δ6-desaturase and the nucleic acids therefor which retain activity for converting LA to GLA.

In another aspect of the present invention, a vector containing a nucleic acid of the present invention or a smaller fragment containing the promoter, coding sequence and termination region of a Δ6-desaturase gene is transferred into an organism, for example, cyanobacteria, in which the Δ6-desaturase promoter and termination regions are functional. Accordingly, organisms producing recombinant Δ6-desaturase are provided by this invention. Yet another aspect of this invention provides isolated Δ6-desaturase, which can be purified from the recombinant organisms by standard methods of protein purification. (For example, see Ausubel et al. [1987] Current Protocols in Molecular Biology, Green Publishing Associates, New York).

Vectors containing DNA encoding Δ6-desaturase are also provided by the present invention. It will be apparent to one of ordinary skill in the art that

appropriate vectors can be constructed to direct the expression of the Δ6-desaturase coding sequence in a variety of organisms. Replicable expression vectors are particularly preferred. Replicable expression vectors as described herein are DNA or RNA molecules engineered for controlled expression of a desired gene, i.e. the Δ6-desaturase gene. Preferably the vectors are plasmids, bacteriophages, cosmids or viruses. Shuttle vectors, e.g. as described by Wolk et al. (1984) Proc. Natl. Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J. Bacteriol. 174, 7525-7533, are also contemplated in accordance with the present invention. Sambrook et al. (1989), Goeddel, ed. (1990) Methods in Enzymology 185 Academic Press, and Perbal (1988) A Practical Guide to Molecular Cloning, John Wiley and Sons, Inc., provide detailed reviews of vectors into which a nucleic acid encoding the present Δ6-desaturase can be inserted and expressed. Such vectors also contain nucleic acid sequences which can effect expression of nucleic acids encoding Δ6-desaturase. Sequence elements capable of effecting expression of a gene product include promoters, enhancer elements, upstream activating sequences, transcription termination signals and polyadenylation sites. The upstream 5' untranslated region of the evening primrose Δ6-desaturase gene as depicted in Figure 10 may also be used. Both constitutive and tissue specific promoters are contemplated. For transformation of plant cells, the cauliflower mosaic virus (CaMV) 35S promoter, other

constitutive promoters and promoters which are regulated during plant seed maturation are of particular interest. All such promoter and transcriptional regulatory elements, singly or in combination, are contemplated for use in the present replicable expression vectors and are known to one of ordinary skill in the art. The CaMV 355 promoter is described, for example, by Restrepo et al. (1990) Plant Cell 2, 987. Genetically engineered and mutated regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine vectors and regulatory elements suitable for expression in a particular host cell. For example, a vector comprising the promoter from the gene encoding the carboxylase of Anabaena operably linked to the coding region of Δ6-desaturase and further operably linked to a termination signal from Synechocystis is appropriate for expression of Δ6-desaturase in cyanobacteria. "Operably linked" in this context means that the promoter and terminator sequences effectively function to regulate transcription. As a further example, a vector appropriate for expression of Δ6-desaturase in transgenic plants can comprise a seed-specific promoter sequence derived from helianthinin, napin, or glycinin operably linked to the Δ6-desaturase coding region and further operably linked to a seed-specific termination signal or the nopaline synthase termination signal. As a still further example, a vector for use in expression of Δ6-desaturase in plants can comprise a constitutive promoter or a tissue specific promoter operably linked

to the Δ6-desaturase coding region and further operably linked to a constitutive or tissue specific terminator or the nopaline synthase termination signal.

In particular, the helianthinin regulatory elements disclosed in applicant's copending U.S. Application Serial No. 682,354, filed April 8, 1991, and incorporated herein by reference, are contemplated as promoter elements to direct the expression of the Δ6-desaturases of the present invention. The albumin regulatory elements disclosed in applicant's copending U.S. application Serial No. 08/831,570 and the oleosin regulatory elements disclosed in applicant's copending U.S. application Serial No.08/831,575 (both applications filed April 9, 1997), and incorporated herein by reference, are also contemplated as elements to direct the expression of the Δ6-desaturases of the present invention.

Modifications of the nucleotide sequences or regulatory elements disclosed herein which maintain the functions contemplated herein are within the scope of this invention. Such modifications include insertions, substitutions and deletions, and specifically substitutions which reflect the degeneracy of the genetic code.

Standard techniques for the construction of such hybrid vectors are well-known to those of ordinary skill in the art and can be found in references such as Sambrook et al. (1989), or any of the myriad of laboratory manuals on recombinant DNA technology that

are widely available. A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments. It is further contemplated in accordance with the present invention to include in the hybrid vectors other nucleotide sequence elements which facilitate cloning, expression or processing, for example sequences encoding signal peptides, a sequence encoding KDEL or related sequence, which is required for retention of proteins in the endoplasmic reticulum or sequences encoding transit peptides which direct Δ6-desaturase to the chloroplast. Such sequences are known to one of ordinary skill in the art. An optimized transit peptide is described, for example, by Van den Broeck et al. (1985) Nature 313, 358. Prokaryotic and eukaryotic signal sequences are disclosed, for example, by Michaelis et al. (1982) Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention provides organisms other than cyanobacteria or plants which contain the DNA encoding the Δ6-desaturase of the present invention. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely

known and provided in references such as Sambrook et al. (1989).

A variety of plant transformation methods are known. The Δ6-desaturase gene can be introduced into plants by a leaf disk transformation-regeneration procedure as described by Horsch et al. (1985) Science 227, 1229. Other methods of transformation, such as protoplast culture (Horsch et al. (1984) Science 223, 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983) Cell 32, 1033) can also be used and are within the scope of this invention. In a preferred embodiment plants are transformed with Agrobacterium-derived vectors such as those described in Klett et al. (1987) Annu. Rev. Plant Physiol. 38:467. However, other methods are available to insert the Δ6-desaturase genes of the present invention into plant cells. Such alternative methods include biolistic approaches (Klein et al. (1987) Nature 327, 70), electroporation, chemically-induced DNA uptake, and use of viruses or pollen as vectors.

When necessary for the transformation method, the Δ6-desaturase genes of the present invention can be inserted into a plant transformation vector, e.g. the binary vector described by Bevan (1984) Nucleic Acids Res. 12, 8111. Plant transformation vectors can be derived by modifying the natural gene transfer system of Agrobacterium tumefaciens. The natural system comprises large Ti (tumor-inducing)-plasmids containing a large segment, known as T-DNA, which is transferred to

transformed plants. Another segment of the Ti plasmid, the *vir* region, is responsible for T-DNA transfer. The T-DNA region is bordered by terminal repeats. In the modified binary vectors the tumor-inducing genes have been deleted and the functions of the *vir* region are utilized to transfer foreign DNA bordered by the T-DNA border sequences. The T-region also contains a selectable marker for antibiotic resistance, and a multiple cloning site for inserting sequences for transfer. Such engineered strains are known as "disarmed" *A. tumefaciens* strains, and allow the efficient transformation of sequences bordered by the T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated with the "disarmed" foreign DNA-containing *A. tumefaciens*, cultured for two days, and then transferred to antibiotic-containing medium. Transformed shoots are selected after rooting in medium containing the appropriate antibiotic, transferred to soil and regenerated.

Another aspect of the present invention provides transgenic plants or progeny of these plants containing the isolated DNA of the invention. Both monocotyledenous and dicotyledenous plants are contemplated. Plant cells are transformed with the isolated DNA encoding Δ6-desaturase by any of the plant transformation methods described above. The transformed plant cell, usually in a callus culture or leaf disk, is regenerated into a complete transgenic plant by methods

well-known to one of ordinary skill in the art (e.g. Horsch et al. (1985) Science 227, 1129). In a preferred embodiment, the transgenic plant is sunflower, oil seed rape, maize, tobacco, peanut or soybean. Since progeny of transformed plants inherit the DNA encoding Δ6-desaturase, seeds or cuttings from transformed plants are used to maintain the transgenic plant line.

The present invention further provides a method for providing transgenic plants with an increased content of GLA. This method includes introducing DNA encoding Δ6-desaturase into plant cells which lack or have low levels of GLA but contain LA, and regenerating plants with increased GLA content from the transgenic cells. In particular, commercially grown crop plants are contemplated as the transgenic organism, including, but not limited to, sunflower, soybean, oil seed rape, maize, peanut and tobacco.

The present invention further provides a method for providing transgenic organisms which contain GLA. This method comprises introducing DNA encoding Δ6-desaturase into an organism which lacks or has low levels of GLA, but contains LA. In another embodiment, the method comprises introducing one or more expression vectors which comprise DNA encoding Δ12-desaturase and Δ6-desaturase into organisms which are deficient in both GLA and LA. Accordingly, organisms deficient in both LA and GLA are induced to produce LA by the expression of Δ12-desaturase, and GLA is then generated due to the expression of Δ6-desaturase. Expression vectors

comprising DNA encoding Δ12-desaturase, or Δ12-desaturase and Δ6-desaturase, can be constructed by methods of recombinant technology known to one of ordinary skill in the art (Sambrook et al., 1989) and the published sequence of Δ12-desaturase (Wada et al [1990] Nature (London) 347, 200-203. In addition, it has been discovered in accordance with the present invention that nucleotides 2002-3081 of SEQ. ID NO:1 encode cyanobacterial Δ12-desaturase. Accordingly, this sequence can be used to construct the subject expression vectors. In particular, commercially grown crop plants are contemplated as the transgenic organism, including, but not limited to, sunflower, soybean, oil seed rape, maize, peanut and tobacco.

The present invention is further directed to a method of inducing chilling tolerance in plants. Chilling sensitivity may be due to phase transition of lipids in cell membranes. Phase transition temperature depends upon the degree of unsaturation of fatty acids in membrane lipids, and thus increasing the degree of unsaturation, for example by introducing Δ6-desaturase to convert LA to GLA, can induce or improve chilling resistance. Accordingly, the present method comprises introducing DNA encoding Δ6-desaturase into a plant cell, and regenerating a plant with improved chilling resistance from said transformed plant cell. In a preferred embodiment, the plant is a sunflower, soybean, oil seed rape, maize, peanut or tobacco plant.

The following examples further illustrate the present invention.

EXAMPLE 1

Strains and Culture Conditions

Synechocystis (PCC 6803, ATCC 27184), Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC 7942, ATCC 33912) were grown photoautotrophically at 30°C in BG11N+ medium (Rippka et al. [1979] J. Gen. Microbiol. 111, 1-61) under illumination of incandescent lamps ($60\mu E.m^{-2}.S^{-1}$). Cosmids and plasmids were selected and propagated in Escherichia coli strain DH5α on LB medium supplemented with antibiotics at standard concentrations as described by Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York.

EXAMPLE 2

Construction of *Synechocystis* Cosmid Genomic Library

Total genomic DNA from *Synechocystis* (PCC 6803) was partially digested with *Sau*3A and fractionated on a sucrose gradient (Ausubel *et al*. [1987] *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, New York). Fractions containing 30 to 40 kb DNA fragments were selected and ligated into the dephosphorylated *Bam*HI site of the cosmid vector, pDUCA7 (Buikema *et al*. [1991] *J. Bacteriol.* 173, 1879-1885). The ligated DNA was packaged *in vitro* as described by Ausubel *et al*. (1987), and packaged phage were propagated in *E. coli* DH5α containing the *Ava*I and *Eco*4711 methylase helper plasmid, pRL528 as described by Buikema *et al*. (1991). A total of 1152 colonies were isolated randomly and maintained individually in twelve 96-well microtiter plates.

EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately $2x10^8$ cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50 µg/ml kanamycin and 17.5 µg/ml chloramphenicol and was subsequently patched onto BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30 µg/ml of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after conjugation and grown in 2 ml BG11N+ liquid medium with 15 µg/ml neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and

transgenic cyanobacterial cultures were harvested by centrifugation and washed twice with distilled water. Fatty acid methyl esters were extracted from these cultures as described by Dahmer et al. (1989) J. Amer. Oil. Chem. Soc. 66, 543-548 and were analyzed by Gas Liquid Chromatography (GLC) using a Tracor-560 equipped with a hydrogen flame ionization detector and capillary column (30 m x 0.25 mm bonded FSOT Superox II, Alltech Associates Inc., IL). Retention times and co-chromatography of standards (obtained from Sigma Chemical Co.) were used for identification of fatty acids. The average fatty acid composition was determined as the ratio of peak area of each C18 fatty acid normalized to an internal standard.

Representative GLC profiles are shown in Fig. 2. C18 fatty acid methyl esters are shown. Peaks were identified by comparing the elution times with known standards of fatty acid methyl esters and were confirmed by gas chromatography-mass spectrometry. Panel A depicts GLC analysis of fatty acids of wild type Anabaena. The arrow indicates the migration time of GLA. Panel B is a GLC profile of fatty acids of transconjugants of Anabaena with pAM542+1.8F. Two GLA producing pools (of 25 pools representing 250 transconjugants) were identified that produced GLA. Individual transconjugants of each GLA positive pool were analyzed for GLA production; two independent transconjugants, AS13 and AS75, one from each pool, were identified which expressed significant levels of GLA and

which contained cosmids, cSy13 and cSy75, respectively (Figure 3). The cosmids overlap in a region approximately 7.5 kb in length. A 3.5 kb NheI fragment of cSy75 was recloned in the vector pDUCA7 and transferred to Anabaena resulting in gain-of-function expression of GLA (Table 2).

Two NheI/Hind III subfragments (1.8 and 1.7 kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3) for sequencing. Standard molecular biology techniques were performed as described by Maniatis et al. (1982) and Ausubel et al. (1987). Dideoxy sequencing (Sanger et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-5467) of pBS1.8 was performed with "SEQUENASE" (United States Biochemical) on both strands by using specific oligonucleotide primers synthesized by the Advanced DNA Technologies Laboratory (Biology Department, Texas A & M University). DNA sequence analysis was done with the GCG (Madison, WI) software as described by Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were transferred into a conjugal expression vector, AM542, in both forward and reverse orientations with respect to a cyanobacterial carboxylase promoter and were introduced into Anabaena by conjugation. Transconjugants containing the 1.8 kb fragment in the forward orientation (AM542-1.8F) produced significant quantities of GLA and octadecatetraenoic acid (Figure 2; Table 2). Transconjugants containing other constructs, either

reverse oriented 1.8 kb fragment or forward and reverse oriented 1.7 kb fragment, did not produce detectable levels of GLA (Table 2).

Figure 2 compares the C18 fatty acid profile of an extract from wild type Anabaena (Figure 2A) with that of transgenic Anabaena containing the 1.8 kb fragment of cSy75-3.5 in the forward orientation (Figure 2B). GLC analysis of fatty acid methyl esters from AM542-1.8F revealed a peak with a retention time identical to that of authentic GLA standard. Analysis of this peak by gas chromatography-mass spectrometry (GC-MS) confirmed that it had the same mass fragmentation pattern as a GLA reference sample. Transgenic Anabaena with altered levels of polyunsaturated fatty acids were similar to wild type in growth rate and morphology.

EXAMPLE 4

Transformation of Synechococcus with Δ6 and Δ12 Desaturase Genes

A third cosmid, cSy7, which contains a Δ12-desaturase gene, was isolated by screening the Synechocystis genomic library with a oligonucleotide synthesized from the published Synechocystis Δ12-desaturase gene sequence (Wada et al. [1990] Nature (London) 347, 200-203). A 1.7 kb AvaI fragment from this cosmid containing the Δ12-desaturase gene was identified and used as a probe to demonstrate that cSy13 not only contains a Δ6-desaturase gene but also a Δ12-desaturase gene (Figure 3). Genomic Southern blot analysis further showed that both the Δ6-and Δ12-desaturase genes are unique in the Synechocystis genome so that both functional genes involved in C18 fatty acid desaturation are linked closely in the Synechocystis genome.

The unicellular cyanobacterium Synechococcus (PCC 7942) is deficient in both linoleic acid and GLA(3). The Δ12 and Δ6-desaturase genes were cloned individually and together into pAM854 (Bustos et al. [1991] J. Bacteriol. 174, 7525-7533), a shuttle vector that contains sequences necessary for the integration of foreign DNA into the genome of Synechococcus (Golden et al. [1987] Methods in Enzymol. 153, 215-231). Synechococcus was transformed with these gene constructs and colonies were selected. Fatty acid methyl esters

were extracted from transgenic Synechococcus and analyzed by GLC.

Table 2 shows that the principal fatty acids of wild type Synechococcus are stearic acid (18:0) and oleic acid (18:1). Synechococcus transformed with pAM854-Δ12 expressed linoleic acid (18:2) in addition to the principal fatty acids. Transformants with pAM854-Δ6 and Δ12 produced both linoleate and GLA (Table 1). These results indicated that Synechococcus containing both Δ12- and Δ6-desaturase genes had gained the capability of introducing a second double bond at the Δ12 position and a third double bond at the Δ6 position of C18 fatty acids. However, no changes in fatty acid composition was observed in the transformant containing pAM854-Δ6, indicating that in the absence of substrate synthesized by the Δ12 desaturase, the Δ6-desaturase is inactive. This experiment further confirms that the 1.8 kb NheI/HindIII fragment (Figure 3) contains both coding and promoter regions of the Synechocystis Δ6-desaturase gene. Transgenic Synechococcus with altered levels of polyunsaturated fatty acids were similar to wild type in growth rate and morphology.

TABLE 2

Composition of C18 Fatty Acids in Wild Type and Transgenic Cyanobacteria

Strain	Fatty acid (%) 18:0	18:1	18:2	18:3(α)	18:3(γ)	18:4
Wild Type						
Synechocystis (sp. PCC6803)	13.6	4.5	54.5	—	27.3	—
Anabaena (sp. PCC7120)	2.9	24.8	37.1	35.2	—	—
Synechococcus (sp. PCC7942)	20.6	79.4	—	—	—	—
Anabaena Transconjugants						
pAM75	3.8	24.4	22.3	9.1	27.9	12.5
pAM75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
pAM542 - 1.8F	4.2	13.9	12.1	19.1	25.4	25.4
pAM542 - 1.8R	7.7	23.1	38.4	30.8	—	—
pAM542 - 1.7F	2.8	27.8	36.1	33.3	—	—
pAM542 - 1.7R	2.8	25.4	42.3	29.6	—	—
Synechococcus Transconjugants						
pAM854	27.8	72.2	—	—	—	—
pAM854 - Δ^{12}	4.0	43.2	46.0	—	—	—
pAM854 - Δ^{6}	18.2	81.8	—	—	—	—
pAM854 - Δ^{6} & Δ^{12}	42.7	25.3	19.5	—	16.5	—

18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3(α), α-linolenic acid; 18:3(γ), γ-linolenic acid; 18:4, octadecatetraenoic acid

EXAMPLE 5

Nucleotide Sequence of Δ6-Desaturase

The nucleotide sequence of the 1.8 kb fragment of cSy75-3.5 including the functional Δ6-desaturase gene was determined. An open reading frame encoding a polypeptide of 359 amino acids was identified (Figure 4). A Kyte-Doolittle hydropathy analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-132) identified two regions of hydrophobic amino acids that could represent transmembrane domains (Figure 1A); furthermore, the hydropathic profile of the Δ6-desaturase is similar to that of the Δ12-desaturase gene (Figure 1B; Wada et al.) and Δ9-desaturases (Thiede et al. [1986] J. Biol. Chem. 261, 13230-13235). However, the sequence similarity between the Synechocystis Δ6- and Δ12-desaturases is less than 40% at the nucleotide level and approximately 18% at the amino acid level.

EXAMPLE 6

Transfer of Cyanobacterial Δ^6-Desaturase into Tobacco

The cyanobacterial Δ^6-desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis Δ-desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive Δ6-desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extension gene or sunflower helianthinin gene to target newly synthesized Δ6-desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the Δ6-desaturase ORF, and (iv) an optimized transit peptide to target Δ6 desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 9, 2145.

Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene, comprised of the Synechocystis Δ6-desaturase gene fused to an endoplasmic reticulum retention sequence (KDEL) and extensin signal peptide driven by the CaMV 35S promoter. PCR amplifications of transgenic tobacco genomic DNA indicate that the Δ6-desaturase gene was incorporated into the tobacco genome. Fatty acid methyl esters of leaves of these transgenic tobacco plants were extracted and analyzed by Gas Liquid Chromatography (GLC). These transgenic tobacco accumulated significant amounts of GLA (Figure 4). Figure 4 shows fatty acid methyl esters as determined by GLC. Peaks were identified by comparing the elution times with known standards of fatty acid methyl ester. Accordingly, cyanobacterial genes involved in fatty acid metabolism can be used to generate transgenic plants with altered fatty acid compositions.

EXAMPLE 7

Construction of Borage cDNA library

Membrane bound polysomes were isolated from borage seeds 12 days post pollination (12 DPP) using the protocol established for peas by Larkins and Davies (1975 Plant Phys. 55:749-756). RNA was extracted from the polysomes as described by Mechler (1987 Methods in Enzymology 152:241-248, Academic Press).

Poly-A+ RNA was isolated from the membrane bound polysomal RNA by use of Oligotex-dT beads (Qiagen). Corresponding cDNA was made using Stratagene's ZAP cDNA synthesis kit. The cDNA library was constructed in the lambda ZAP II vector (Stratagene) using the lambda ZAP II vector kit. The primary library was packaged in Gigapack II Gold packaging extract (Stratagene). The library was used to generate expressed sequence tags (ESTs), and sequences corresponding to the tags were used to scan the GenBank database.

EXAMPLE 8

Hybridization Protocol

Hybridization probes for screening the borage cDNA library were generated by using random primed DNA synthesis as described by Ausubel et al (1994 Current Protocols in Molecular Biology, Wiley Interscience, N.Y.) and corresponded to previously identified abundantly expressed seed storage protein cDNAs. Unincorporated nucleotides were removed by use of a G-50 spin column (Boehringer Manheim). Probe was denatured for hybridization by boiling in a water bath for 5 minutes, then quickly cooled on ice. Filters for hybridization were prehybridized at 60°C for 2-4 hours in prehybridization solution (6XSSC [Maniatis et al 1984 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory], 1X Denharts Solution, 0.05% sodium pyrophosphate, 100 µg/ml denatured salmon sperm DNA). Denatured probe was added to the hybridization solution (6X SSC, 1X Denharts solution, 0.05% sodium pyrophosphate, 100 µg/ml denatured salmon sperm DNA) and incubated at 60°C with agitation overnight. Filters were washed in 4x, 2x, and 1x SET washes for 15 minutes each at 60°C. A 20X SET stock solution is 3M NaCl, 0.4 M Tris base, 20 mM $Na_2EDTA\text{-}2H_2O$. The 4X SET wash was 4X SET, 12.5 mM PO_4, pH 6.8 and 0.2% SDS. The 2X SET wash was 2X SET, 12.5 mM PO_4, pH 6.8 and 0.2% SDS. The 1X SET wash was 1X SET, 12.5 mM PO_4, pH 6.8 and 0.2% SDS. Filters were allowed to air dry and were then exposed to X-ray film for 24 hours with intensifying screens at -80°C.

EXAMPLE 9

Random sequencing of cDNAs from a borage seed (12 DPP) membrane-bound polysomal library

The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the Δ6-desaturase were identified.

Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis Δ6-desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks (IntelligGenetics) protein alignment program

(Fig. 2). This alignment indicated that the cDNA was the borage Δ6-desaturase gene.

Although similar to other known plant desaturases, the borage delta 6-desaturase is distinct as indicated in the dendrogram shown in Fig. 6. Furthermore, comparison of the amino acid sequences characteristic of desaturases, particularly those proposed to be involved in metal binding (metal box 1 and metal box 2), illustrates the differences between the borage delta 6-desaturase and other plant desaturases (Table 3).

The borage delta 6-desaturase is distinguished from the cyanobacterial form not only in over all sequence (Fig. 6) but also in the lipid box, metal box 1 and metal box 2 amino acid motifs (Table 3). As Table 3 indicates, all three motifs are novel in sequence. Only the borage delta 6-desaturase metal box 2 showed some relationship to the *Synechocystis* delta-6 desaturase metal box 2.

In addition, the borage delta 6-desaturase is also distinct from another borage desaturase gene, the delta-12 desaturase. P1-81 is a full length cDNA that was identified by EST analysis and shows high similarity to the *Arabidopsis* delta-12 desaturase (Fad 2). A comparison of the lipid box, metal box 1 and metal box 2 amino acid motifs (Table 3) in borage delta 6 and delta-12 desaturases indicates that little homology exists in these regions. The placement of the two sequences in the dendrogram in Fig. 6 indicates how distantly related these two genes are.

Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Amino Acid Motif: Lipid Box	Metal Box 1	Metal Box 2
Borage Δ^6	WIGHDAGH (SEQ. ID. NO: 6)	HNAHH (SEQ. ID. NO: 12)	FQIEHH (SEQ. ID. NO: 20)
Synechocystis Δ^6	NVGHDANH (SEQ. ID. NO: 7)	HNYLHH (SEQ. ID. NO: 13)	HQVTHH (SEQ. ID. NO: 21)
Arab. chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Rice Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Glycine chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Arab. fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Brassica fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)
Borage Δ^{12} (P1-81)*	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)
Arab. fad2 (Δ^{12})	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)
Arab. chloroplast Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)
Glycine plastid Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO: 10)	HDQHH (SEQ. ID. NO: 17)	HIPHH (SEQ. ID. NO: 24)
Synechocystis Δ^{12}	VVGHDCGH (SEQ. ID. NO: 11)	HDHHH (SEQ. ID. NO: 18)	HIPHH (SEQ. ID. NO: 24)
Anabaena Δ^{12}	VLGHDCGH (SEQ. ID. NO: 8)	HNHHH (SEQ. ID. NO: 19)	HVPHH (SEQ. ID. NO: 25)

*P1-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arbidopsis Δ12 desaturase (fad2)

EXAMPLE 10

Construction of 222.1Δ^6NOS for transient and expression

The vector pBI221 (Jefferson et al. 1987 EMBO J. 6:3901-3907) was prepared for ligation by digestion with BamHI and EcoICR I (Promega) which excises the GUS coding region leaving the 35S promoter and NOS terminator intact. The borage Δ6-desaturase cDNA was excised from the Bluescript plasmid (Stratagene) by digestion with BamHI and XhoI. The XhoI end was made blunt by use of the Klenow fragment. This fragment was then cloned into the BamHI/EcoICR I sites of pBI221, yielding 221.1Δ^6NOS (Fig. 7). In 221.1Δ^6.NOS, the remaining portion (backbone) of the restriction map depicted in Fig. 7 is pBI221.

EXAMPLE 11

Construction of $121.1\Delta^{6}$.NOS for stable transformation

The vector pBI121 (Jefferson et al. 1987 EMBO J. 6:3901-3907) was prepared for ligation by digestion with BamHI and EcoICR I (Promega) which excises the GUS coding region leaving the 35S promoter and NOS terminator intact. The borage Δ6-desaturase cDNA was excised from the Bluescript plasmid (Stratagene) by digestion with BamHI and XhoI. The XhoI end was made blunt by use of the Klenow fragment. This fragment was then cloned into the BamHI/EcoICR I sites of pBI121, yielding $121.1\Delta^{6}$NOS (Fig. 7). In $121.\Delta^{6}$.NOS, the remaining portion (backbone) of the restriction map depicted in Fig. 7 is pBI121.

EXAMPLE 12

Transient Expression

All work involving protoplasts was performed in a sterile hood. One ml of packed carrot suspension cells were digested in 30 mls plasmolyzing solution (25 g/l KC1, 3.5 g/l $CaCl_2$-H_2O, 10mM MES, pH 5.6 and 0.2 M mannitol) with 1% cellulase, 0.1% pectolyase, and 0.1% dreisalase overnight, in the dark, at room temperature. Released protoplasts were filtered through a 150 µm mesh and pelleted by centrifugation (100x g, 5 min.) then washed twice in plasmolyzing solution. Protoplasts were counted using a double chambered hemocytometer. DNA was transfected into the protoplasts by PEG treatment as described by Nunberg and Thomas (1993 Methods in Plant Molecular Biology and Biotechnology, B.R. Glick and J.E. Thompson, eds. pp. 241-248) using 10^6 protoplasts and 50-70 ug of plasmid DNA (221.Δ6.NOS). Protoplasts were cultured in 5 mls of MS media supplemented with 0.2M mannitol and 3 µm 2,4-D for 48 hours in the dark with shaking.

EXAMPLE 13

Stable transformation of tobacco

121.1Δ^6NOS plasmid construction was used to transform tobacco (Nicotiana tabacum cv. xanthi) via Agrobacterium according to standard procedures (Horsh et al., 1985 Science 227: 1229-1231; Bogue et al., 1990 Mol. Gen. Genet. 221:49-57), except that initial transformants were selected on 100 ug/ml kanamycin.

EXAMPLE 14

Preparation and analysis of fatty acid methyl esters (FAMEs)

Tissue from transfected protoplasts and transformed tobacco plants was frozen in liquid nitrogen and lyophilized overnight. FAMEs were prepared as described by Dahmer et al (1989 J. Amer. Oil Chem. Soc. 66:543-548). In some cases, the solvent was evaporated again, and the FAMEs were resuspended in ethyl acetate and extracted once with deionized water to remove any water soluble contaminants. The FAMEs were analyzed by gas chromatography (GC) on a J&W Scientific DB-wax column (30 m length, 0.25 mm ID, 0.25 um film).

An example of a transient assay is shown in Fig. 8 which represents three independent transfections pooled together. The addition of the borage Δ6-desaturase cDNA corresponds with the appearance of gamma linolenic acid (GLA) which is one of the possible products of Δ6-desaturase. Furthermore, transgenic tobacco containing the borage Δ6-desaturase driven by the cauliflower mosaic virus 35S promoter also produce GLA as well as octadecaenoic acid (18:4) which is formed by the further desaturation of GLA (Fig. 9). These results indicate that the borage delta 6-desaturase gene can be used to transform plant cells to achieve altered fatty acid compositions.

EXAMPLE 15

Isolation of an Evening Primrose Δ6-desaturase gene

Total RNA was isolated from evening primrose embryos using the method of Chang, Puryear, and Cairney (1993) *Plant Mol Biol Reporter 11*:113-116. Poly A^+ RNA was selected on oligotex beads (Qiagen) and used as a template for cDNA synthesis. The cDNA library was constructed in the lambda ZAP II vector (Stratagene) using the lambda ZAP II vector kit. The primary library was packaged with Gigapack II Gold packaging extract (Stratagene).

PCR primers based on sequences in the borage Δ6-desaturase gene were synthesized by a commercial source using standard protocols and included the following oligonucleotides:

5' AAACCAATCCATCCAAGRA 3' SEQ ID NO:27

5' KTGGTGGAAATGGAMSCATAA 3' SEQ ID NO:28

(R=A and G, K=G and T, M=A and C, S=G and C)

A primer that matches a region that flanks the insertion site of the lambda ZAP II vector was also synthesized using an ABI394 DNA synthesizer and standard protocols. This primer ahd the following sequence:

5' TCTAGAACTAGTGGATC 3' SEQ ID NO:29

An aliquot of the cDNA library was used directly as template in a PCR reaction using SEQ ID NO: 27 and SEQ ID NO:29 as primers. The reactions were carried out in a volume of 50 µl using an annealing temperature of 50°C for 2 minutes, an

extension temperature of 72°C for 1.5 minutes, and a melting temperature of 94°C for 1 minute for 29 cycles. A final cycle with a 2 minute annealing at 50°C and a 5 minute extension at 72°C completed the reaction. One µl from this reaction was used as a template in a second reaction using the same conditions except that the primers were SEQ ID NO:27 and SEQ ID NO:28. A DNA fragment of predicted size based on the location of the primer sequences in the the borage Δ6-desaturase cDNA was isolated.

This PCR fragment was cloned into pT7 Blue (Novagen) and used to screen the evening primrose cDNA library at low stringency conditions: The hybridization buffer used was 1% bovine serum albumin (crystalline fraction V), 1mM EDTA, 0.5 M $NaHPO_4$ pH7.2, and 7% SDS. The hybridizations were at 65°C. The wash buffer was 1mM Na_2EDTA, 40 mM $NaHPO_4$pH7.2 and 1% SDS. Primary screens were washed at 25°C. Secondary and tertiary screens were washed at 25°C, 37°C, and 42°C. One of the positively hybridizing clones that was identified in the evening primrose cDNA library was excised as a phagemid in pBluescript. The DNA sequence of the 1687 bp insert of this phagemid (pIB9748-4) was determined (Fig. 10, SEQ ID NO: 26) using the ABIPRISM™ dye terminator cycle sequencing core kit from Perkin Elmer according to the manufacturer's protocol. The sequence encodes a full length protein of 450 amino acids (SEQ ID NO:27) with a molecular weight of 51492 daltons.

Alignment of the deduced amino acid sequence with that of borage Δ6-desaturase was performed using

the Geneworks program (Fig. 11). The evening primrose Δ6-desaturase protein is identical at 58% of the residues and similar at an additional 20% of the residues. Only two small gaps, near the carboxy terminal end of the protein were introduced by the program to obtain the alignment (Fig. 11). The two proteins were compared using two different alogorithms that measure the hydrophobicity of regions to the protein. Figures 12A and 12B are Kyte-Doolittle hydrophobicity plots of borage Δ6-desaturase and evening primrose Δ6-desaturase, respectively. Figures 13A and 13B are Hopwood hydrophobicity plots generated in the program DNA Strider for the same proteins. A discussion of the algorithim used to generate these plots can be found in Hopp, T.P. and Woods, K.R. 1983 Molecular Immunology 20:483-89. Substantial similarity exists between the borage and evening primrose proteins using either algorithm. TMPredict, a program that predicts the location of transmembrane regions of proteins was run on the two sequences and again similar results were obtained (Figures 14 and 15). Several weights matrices are used in scoring the predictions as reported in Hofmann, K. and Stoffel, W. 1993 *Biol. C. Hoppe-Scyler 347:156.* Positive values (x-axis) greater than 500 are considered significant predictors of a membrane spanning region; the x-axis represents the linear amino acid sequences.

The membrane bound desaturases of plants possess three histidine rich motiffs (HRMs). These motiffs are identified in the evening primrose

sequence and are indicated in Figure 10 by underlined plain text. The motifs in this sequence were identical to those found in borage Δ6-desaturase with the exception of those that are italicized (S 161 and L374). The borage Δ6-desaturase is unique among known membrane bound desaturases in having a cytochrome *b*5 domain at the carboxy terminal end. The evening primrose protein encoded by pIB9748-4 also has this domain. The heme binding motiff of chtochrome *b*5 proteins is indicated in Figure 10 by underlined bold text.

These data indicate that a Δ6-desaturase cDNA from evening primrose has been isolated and characterized.

EXAMPLE 16

Construction of expression vectors for transient and stable expression of an evening primrose Δ6-desaturase

The evening primrose Δ6-desaturase cDNA is excised from the Bluescript phagemid by digestion with Xba I and Xho I. The entire cDNA sequence including the 5' transcribed but untranslated region depicted in Figure 10 (SEQ ID NO:26) is operably linked to any one of various promoters and/or other regulatory elements in an expression vector, in order to effect transcription and translation of the Δ6-desaturase gene. Alternatively, the cDNA sequence depicted in Figure 10 may be trimmed at the 5' end so that the 5' transcribed but untranslated sequence is removed. The A of the ATG translational start codon is then made the first nucleotide following the promoter and/or other regulatory sequence in an expression vector.

In order to express the subject evening primrose cDNA in pBI221 (Jefferson et al. 1987 EMBO J. 6:3901-3907) the following manipulations are performed:

The plasmid pBI221 is digested with EcoICR I (Promega) or Ecl 136 II (NEB) and Xba I which excises the GUS coding region and leaves the 35S promoter and NOS terminator intact. The evening primrose Δ6-desaturase cDNA is excised from pIB9748-4 by digestion with Xba I and Xho I. The Xho I end is made blunt by use of the Klenow fragment. The excised gene is then cloned into the cloned into the Xba I/Eco ICR I sites of pBI221. The resulting construct is then

transfected into carrot protoplasts. One ml of packed carrot suspension cells are digested in 30 ml of plasmolyzing solution (25 g/l KCl 3.5 g/l $CaCl_2$-H_2O, 10 mM MES, pH 5.6 and 0.2 M mannitol) with 1% cellulase 0.1% pectolyase, and 0.1% dreisalase overnight, in the dark, at room temperature. Released protoplasts are filtered through a 150 µm mesh and pelleted by centrifugation (100 x g, 5 minutes), then washed twice in plasmolyzing solution. Protoplasts are counted using a double chambered hemocytometer. DNA is transfected into the protoplasts by PEG treatment as described by Nunberg and Thomas (1993 Methods in Plant Molecular Biology and Biotechnology, B.R. Glick and J.E. Thompson, eds. pp 241-248) using 10^6 protoplasts and 50-70 ug of DNA from the above construct. Protoplasts are cultured in 5 ml of MS medium supplemented with 0.2 M mannitol and 3 µM 2, 4-D for 48 hours in the dark with shaking. Tobacco is transformed with the same Δ6-desaturase expression construct by following the method of Example 13.

In order to express the subject evening primrose cDNA in pBI121 (Jefferson et al. 1987 EMLBO J. 6:3901-3907), the following manipulations are performed:

The plasmid pBI121 is digested with EcoICR I (Promega) or Ecl 136 II (NEB) and Xba I which excises the GUS coding region and leaves the 35S promoter and NOS terminator intact. The evening primrose Δ6-desaturase cDNA is excised from pIB9748-4 by digestion with Xba I and Xho I. The Xho I end is made blunt by use of the Klenow fagment. The excised gene is then

cloned into the Xba I/Eco ICR I sites of pBI121. The resulting construct is used to transform *Arabidopsis thaliana* via *Agrobacterium* according to standard protocols (Bechtold N., Ellis. J., and Pelletier, G 1993 C.R. Acad Sci Paris 316:1194-1199). Carrot and tobacco are transformed as described above.